IDENTIFICATION OF CHEMICAL MARKERS FOR BACTERIA BY PYROLYSIS-TANDEM MASS SPECTROMETRY

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INTRODUCTION

There is a substantial body of literature concerning the identification of microorganisms by mass spectrometry (1-11), however, little has been done to identify bacteria in complex variable backgrounds. Several sample introduction and ionization methods have been used for mass-spectrometric analysis of bacteria. Sampling methods may be classified as either direct or indirect, depending on whether whole organisms or extracts are used in the analysis. Of the direct sampling methods, pyrolysis is the most frequently used, although recently FAB methods utilizing whole cells have been shown to be useful for identifying microorganisms (12-13). Pyrolysis has been used with direct introduction of pyrolysate to the ionizer (Py-MS, i.e., pyrolysis *in vacuo*) and also in conjunction with gas chromatography (Py-GCMS). Both electron ionization and chemical ionization have been used in Py-MS and Py-GCMS analyses.

The simplest and most rapid of these methods is direct Py-MS with electron ionization. The Py-mass spectra, however, are generally quite complex and do not easily lend themselves to chemical interpretation. On the other hand, Py-GCMS allows identification of the chemical components of cellular material, hence, specific biomarkers may be found (1,10,14). The presence of such compounds may be used to unambiguously identify target materials, even in complex backgrounds.

Tandem mass spectrometry adds a dimension to traditional Py-MS which permits identification of specific compounds in a pyrolysate (5,11). The purpose of the present research was to determine the feasibility of using Py-MS/MS to detect bacterial biomarkers in complex backgrounds of particulate material.

EXPERIMENTAL

Measurements were obtained with an Extrel Model EL-400 triple quadrupole mass spectrometer fitted with a Curie-point pyrolysis inlet (11). Daughter ion spectra were collected for m/z 79, 111, 117, 126, and 135. These ions were chosen based on our experience with the samples. The samples used are described in Table 1. All samples were suspended in methanol at about 1mg/ml. Approximately 10 μL of each suspension was applied to 610°C Curie-point wires, and the solvent evaporated under a stream of hot air.

Multivariate statistical analyses were carried out with the RESOLVE program (15). Each Py-MS spectrum was collected as a set of raw intensities. Prior to principal components analysis, the data were normalized to constant length. The results, displayed as Karhunen-Loeve (K-L) or principal components plots, show the distribution of the samples in the multivariate mass spectral space. Factor spectra for the components were also derived. Factor spectra show the correlations (positive and negative) of mass spectral peaks with directions in the principal component space.

RESULTS AND DISCUSSION

In previous experiments (unpublished), we found that the Py-MS patterns of bacteria could vary more within strains of a given species than between different species. For example, the intensities of m/z 52 and 79 varied drastically among strains of *B. subtilis* and *B. licheniformis*. Strains showing large m/z 79 and 52 peaks were sporulated, while other strains were vegetative cells. We postulated that the m/z 79 and 52 peaks were markers for sporulation. The daughter ion spectra of m/z 79 for the test samples (Figure 1) showed that m/z 52 is a strong daughter of m/z 79 for the bacteria, while samples such as fog oil and diesel smoke showed virtually no m/z 52 as a daughter of m/z 79.

The principal components plot shown in Figure 2a separates the *B.g.* spores and *B. subtilis* from the rest of the samples. The factor spectrum generated for principal component 2 (Figure 2b) shows peaks at m/z 52 and 79 positively correlated with the *B.g.* and *B. subtilis* samples, while peaks at m/z 51 and 77 are positively correlated with the remainder of the samples.

Sporulating bacteria produce large quantities of picolinic acid (2-pyridine-carboxylic acid) which forms the hardened "shell" of the spores. The EPA/NIH library spectrum (16) of picolinic acid (virtually identical to that of pyridine) shows large peaks at m/z 79 (i.e., pyridine molecular ion) and m/z 52. Because the mass spectra of pyridine and picolinic acid are so similar, it is not possible to determine which of these species is liberated upon pyrolysis. It is clear, however, that the presence of picolinic acid in the spores is responsible for the separation seen in the K-L plot.

The negative correlation of peak m/z 77 with the sporulated organisms indicates that this peak is more intense (relatively) in the other samples as is obvious by inspection of Figure 1. In order to determine the chemical species responsible for the peak at m/z 79 in samples other than sporulated bacteria, parent ion spectra of m/z 79 were collected. The parent ion spectrum of m/z 79 for diesel smoke is shown in Figure 3. This spectrum is quite complicated and it appears that at least three compound classes are present. The peak at m/z 94 appears to be due to alkylcyclodienyl hydrocarbons (e.g., methylcyclohexadiene). The m/z 105 peak may be due to alkyl benzaldehydes (e.g., 2,6 dimethylbenzaldehyde) and the series of peaks at m/z 107, 121, 135 and 149 are probably produced by a series of alkyl phenols. Although these identifications are tentative (based on a comparison of EPA/NIH library spectra with the parent and daughter spectra) each of the compound types listed above show a significant m/z 77 peak as well as an m/z 79

peak. It is clear that many compounds contribute to the peak at m/z 79, however, sporulated bacteria are easily identified by the presence of large quantities of picolinic acid, as reflected in the intense m/z 52 peak in the daughter spectrum of m/z 79.

Representative spectra of daughter ions of m/z 117 are illustrated in Figure 4. The major peaks appearing in these spectra are m/z 89, 80, 91, 115 and 117. The K-L plot in Figure 5a shows scores of the samples on the first two principal components. The fog oil, wood smoke, and diesel smoke samples are clearly separated from the other samples, all of which contain protein. The factor spectrum associated with this separation (Figure 5b) shows two distinctive patterns; one correlated with the fog oil and smokes shows large peaks at m/z 91 and 115 while the other pattern (negative direction) has strong peaks at m/z 89 and 90. The spectrum in the negative direction is that of indole, a known pyrolysis product of proteins containing the amino acid tryptophane. The peaks at m/z 91 and 115 are probably due to unsaturated alkyl aromatic compounds, for example, 2-methylbutenylbenzene. The daughter ion spectrum of m/z 117 appears to contain markers for the presence of protein in a sample.

We have observed that the base adenine (MW 135) is liberated by pyrolysis of DNA. It was postulated that adenine could be used as a marker for organisms since all living material contains nucleic acids. The daughter ion spectra in Figure 6 are representative of the sample set. The K-L plot in Figure 7a shows a distribution of samples in the Component 2 direction with wood smoke, fog oil, and diesel smoke at one extreme and *E. coli* and MS-2 coliphage at the other. The factor spectrum associated with Component 2 (Figure 7b) indicates the presence of adenine (m/z 108) in the negative direction, and a series of alkyl phenols (m/z 107, 93, 79, etc.) in the positive direction. The parent ion scan of m/z 135 for diesel smoke shows a homologous series of alkyl chain lengths (of an alkyl phenol) extending up to at least 290 amu (a C14 alkyl group).

The daughter ion spectrum of m/z 135 indicates the presence of nucleic acids by the appearance of an m/z 108 peak. This suggests that the daughter ion spectrum of m/z 135 may be used to determine the presence of living material (or recently living material) in an aerosol sample. This would provide a useful branch point for a decision tree; if adenine is not present, there is no bacterial threat.

Daughter ion spectra were also collected for m/z 111, 126, and 128. In each case there were clear separations of background materials (i.e., fog oil, diesel and wood smoke) from the targets (i.e., MS-2 coliphage, *E. coli, B.g.* spores, etc.). The factor spectra associated with these separations, however, were quite complex and could not be interpreted chemically with a high degree of confidence. For example, factor spectra for the daughters of m/z 126 showed correlations of peaks with fog oil that indicated the presence of unsaturated hydrocarbons (probably nonene). The spectra associated with other samples, however, could contain contributions from nucleic acids (thymine), proteins, and carbohydrates (methylhydroxypyranone).

CONCLUSIONS

The feasibility of using pyrolysis-tandem mass spectrometry to identify biomarkers in complex backgrounds has been demonstrated. Three markers for biological substances have been identified. Adenine, a marker for nucleic acids and hence all living material, is detected in the daughter ion spectrum of m/z 135. The presence of protein in a sample may be inferred from the daughter ion spectrum of m/z 117, which indicates the presence or absence of indole, a product of pyrolysis of proteins containing the amino acid tryptophane. Pyridine, from picolinic acid, is seen in the daughter ion spectrum of m/z 79. This is a marker for the presence of sporulated bacteria. These ions obviously do not solve the problem of identifying specific organisms in ambient samples. There are however hundreds more ions to be studied, and if biomarkers are present in the pyrolysates, Py-MS/MS has the capability of finding them.

REFERENCES

1. Fox, Alvin; Morgan, Stephen L.; Larsson, Lennart; and Odham, Goran; Eds.; Analytical Microbiology Methods. 1989 Plenum Press, NY.

2. Anhalt, John P. and Fenselau, Catherine, Anal. Chem. 1975, 47(2), 219-255.

3. Voorhees, Kent J.; Harrington, Peter B.; Street, Thomas E.; Hoffman, Stephen; Durfee, Steven L.; Bonelli, Joseph E. and Firnhaber, Cynthia S. from "Computer-Enhanced Analytical Spectroscopy, V. 2, H.L.C. Meuzelaar, Ed., 1990, 257-275.

4. Voorhees, Kent J.; Durfee, Steven L.; and Updegraff, David M., J. Microbiol. Meth. 1988, 8, 315-325.

5. Voorhees, Kent J.; Durfee, Steven L.; Holtzclaw, James R.; Enke, C.G.; and Bauer, Mark R., J. Anal. Appl. Pyrol., 1988, 14, 7-15.

6. Tas, A.C.; Van Der Greef, J.; DeWaart, J.; Bouwman, J.; and Teu Noever de Braw, M.D., J. Anal. Appl. Pyrol., 1985, 7, 249-255.

7. Tas, A.C.; DeWaart, J.; Bouwman, M.C.; Teu Noever de Braw, M.C.; and Van Der Greef, J. J. Anal. Appl. Pyrol., 1987, 11, 329-340.

8. Van der Greef, J.; Tas, A.C.; and Teu Noever de Brauw, M.C. Biomed. Env. Mass Spec., 1988, 16, 45-50.

9. Tas, A.C.; Bastiaanse, H.B.; Van Der Greef, J.; and Kerkenaar, A. J. Anal. Appl. Pyrol., 1989, 14, 309-321.

10. Morgan, Stephen L.; Fox, Alvin; and Gilbert, James. J. Microbiol. Meth., 1989, 9, 57-69.

11. DeLuca, Stephan; Sarver, Emory W.; Harrington, Peter de B.; and Voorhees, Kent J. Anal. Chem. 1990, 62, 1465-1472.

12. Heller, D.N.; Feuselau, C.; Cotter, R.J.; Demirev, P.; Olthoff, J.K.; Honovich, J.; Uy, M.; Tanaka, T. and Kishimoto, Y. Biochem. Biophys. Res. Comm., 1987, 142(1), 194-199.

13. Seid, Robert C., Jr.; Bone, William M.; and Phillips, Lawrence R. Anal. Biochem., 1986, 155, 168-176.

14. Holzer, G.; Bourne, T.F.; Bertsch, W. J. Chromatog. 1989, 468, 181-190.

15. Harrington, P. de B.; Street, T.E.; Voorhees, K.J.; Radicati di Brozolo, F.; Odom, R.W. Anal. Chem. 1989, 61, 715-719.

16. Heller, S.R. and Milne, G.W.A. EPA/NIH Mass Spectral Data Base, 1978, Nat. Stand. ref. data ser.; NSRDS-NBS 63.

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Table 1. Samples used in the study

Substance	Category
Fog oil	f
Wood smoke	w
Diesel smoke	d
Grass pollen (*Secale cerale*)	p
Dry Yeast	y
Aldolase	a
MS-2 coliphage	m
E. coli	e
B. subtilis (sporulated)	b
B. globigii spores (*B. subtilis var. niger*)	g

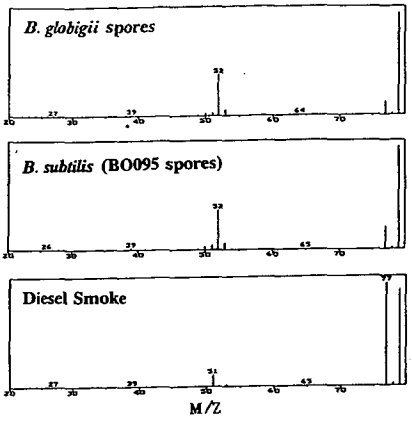

Figure 1. Selected daughter ion spectra for m/z 79.

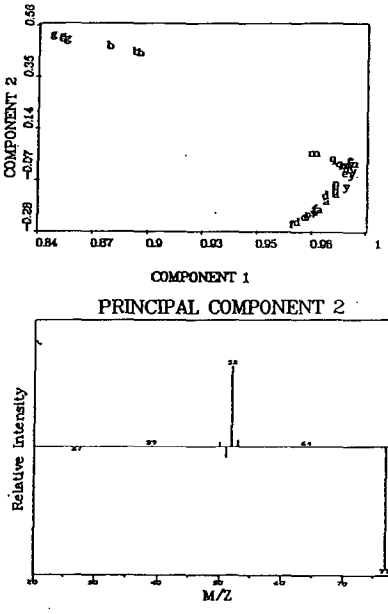

Figure 2. Principal components plot (a) and factor spectrum (b) for daughters of m/z 79.

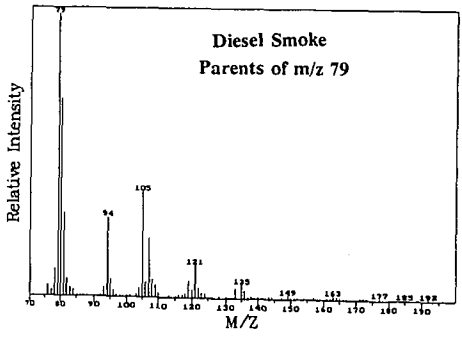

Figure 3. Parent ion spectrum of diesel smoke for m/z 79.

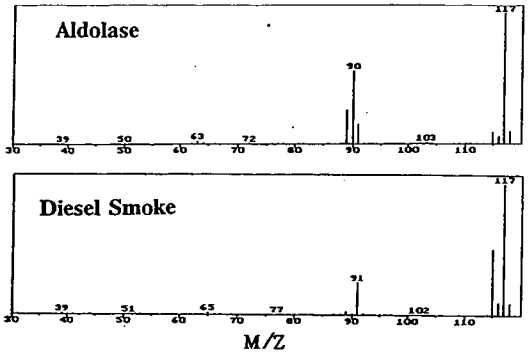

Figure 4. Selected daughter ion spectra for m/z 117

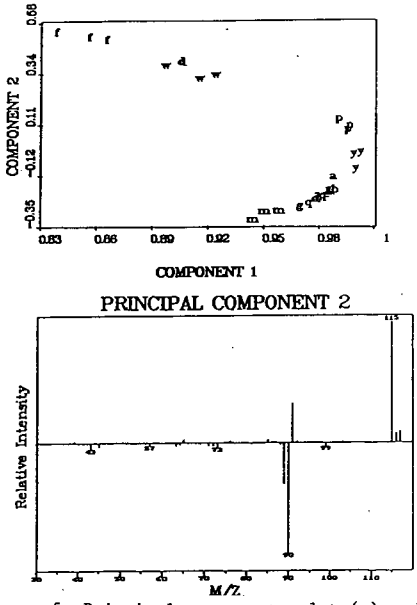

Figure 5. Principal components plot (a) and factor spectrum (b) for daughters of m/z 117.

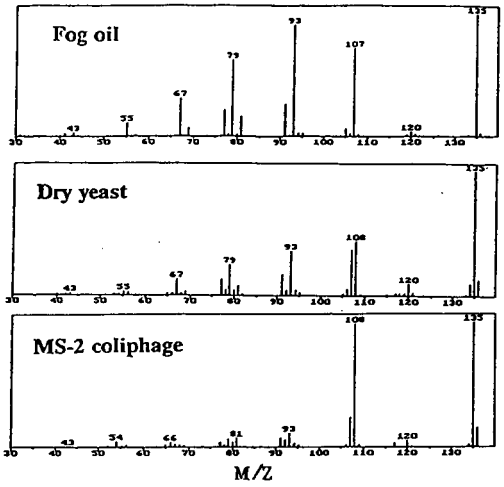

Figure 6. Selected daughter ion spectra for m/z 135.

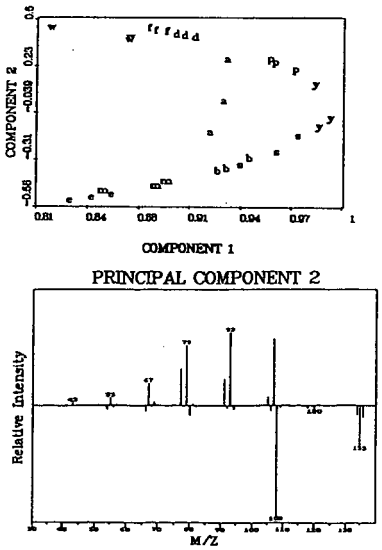

Figure 7. Principal components plot (a) and factor spectrum (b) for daughters of m/z 135